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13. ABSTRACT (Maximum 200 Words)

A novel system for studying growth of normal human mammary epithelium in vivo as grafts in athymic nude mice has been developed. The key feature of this model is the reconstitution of the epithelial-stromal interactions required for normal growth and differentiation of the human mammary epithelium, which produces ducts that are comparable to the normal human mammary gland. Human breast epithelial organoids were combined with mammary fibroblasts from mouse or human origin in collagen gels, which were subsequently transplanted under the renal capsule of female nude mice hosts. The resulting grafts showed an increase in the ductal density than observed previously. These ducts expressed appropriate markers for luminal and myoepithelial cells and steroid receptors. This model allows for a variety of epithelial and stromal cells to be used in combination, which would aid in understanding key factors that regulate normal human mammary gland development. The manuscript detailing this data has been accepted for publication in the December 2002 issue of Endocrinology. The experiments currently in progress utilize both normal and tumor epithelium and carcinoma associated fibroblasts (CAF) in this model to assess the effect of CAF on the development of normal human mammary epithelium. CAF from a variety of tumors including ErbB2 positive tumors will be used.

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INTRODUCTION:

The main focus of this research is studying the epithelial-stromal interactions that take place in the normal and the tumor mammary gland. To aid in this study we have developed a new model to study human mammary gland development in vivo using human breast organoids from reduction mammoplasty specimens combined with fibroblasts from either the mouse or the human mammary gland. The recombinations are placed in a collagen gel, which is then grafted under the renal capsule of female nude mice. Using this strategy has resulted in ductal development resembling the normal human mammary gland. This model has also been used to assess the effect of using tumor fibroblasts in combination with the organoids to test whether they cause abnormal ductal development. Data suggests that mammary CAF does have an effect on breast epithelial development. This model provides a very powerful method in which to study normal mammary gland development and to study the changes that initiate tumor formation.

BODY:

Technical objective 1: Profile ESX expression in normal developmentally staged human breast epithelium.

Using both in situ hybridization and immunohistochemistry, ESX expression was determined in the sections of human breast epithelium. The expression was found in the epithelial cells of the ducts. Since there is still no ESX antibody available that is suitable for immunohistochemistry, this part of the project will be terminated until such a reagent becomes available. To explore other methods of determining ESX expression in malignant and non-malignant breast epithelial cells, I have collaborated on a related project analyzing signaling and transcription factors resulting in ESX promoter activation and transcriptional expression. A manuscript describing our progress on this related project is currently in preparation (Neve et al., 2003).

Technical objective 2.1: confirm that embryonic mammary mesenchyme is capable of inducing a mammary specific pattern of ESX expression.

Embryonic mammary mesenchyme and mammary epithelium were recombined and placed under the renal capsule of female nude mice (Cunha et al., 1995) where it developed into phenotypically normal mouse mammary gland. The gland was removed at various stages throughout the reproductive cycle; virgin, mid pregnant, late pregnant, lactation and involution. Formalin-fixed tissue blocks containing the glandular outgrowths at these different stages of mammary gland developmental have been prepared and are being stored until a suitable anti-ESX antibody becomes available for determining cellular ESX expression (cf. Technical Objective 1). Effort spent on this technical objective is now being redirected toward the additional studies included and described under Technical objective 2.2.

Technical objective 2.2: testing the correlation between stromal age and tumor induction on epithelial ESX and ErbB2 expression.

Work has been initiated to study age-related effects on mammary gland development. I have obtained organoids from reduction mammoplasty specimens (from J. Emerman)

from women whose age ranges from 20-50. These samples will be combined with mammary fibroblasts of a known and consistent passage number. To test the effects of the aged stroma, mammary fibroblasts have been cultured in vitro for up to 20+ generations (towards Hayflick limit and senescence). These have already been recombined with organoids and initial results show abnormal development. Since aging is thought to be accompanied by oxidative damage which may induce either cellular senescence or a preneoplastic phenotype, a new set of studies were initiated taking advantage of the local availability of SOD2 knockout mice (Melov et al., 1999). Manganese superoxide dismutase (also referred to as SOD2) is the primary antioxidant enzyme in the mitochondria that plays a key role in the detoxification of superoxide free radicals and protects cells from oxidative stress. SOD2 knockout mice die of accelerated cardiac and neural aging within a week of birth, making the study of their mammary gland development impossible. However, by resecting the neonatal mammary gland tissue from these newborn mice and implanting it under the renal capsule of female nude mice, we are now able to observe the effects of oxidative stress and accelerated aging on mammary gland growth and hormone induced development. Preliminary results from these studies indicate that the SOD2 knockout mammary glands exhibit significant morphologic abnormalities (hyperplastic, disorganized) epithelial development. Immunohistochemical studies are ongoing to further define these abnormalities.

Technical objective 2.3: Employ ESX-null epithelium to demonstrate its function in mesenchyme-induced mammary gland development.

Despite diligent attempts, we have been unable to secure ESX-null mammary gland tissue or mice from the Australian investigators who recently developed, patented, and described this mouse model. Thus, we have attempted to create ESX null mutants using pronuclear microinjection methodology. Our results have not been successful so far but a new strategy is being utilized in collaboration with other groups. Given that this is now a long term alternative strategy, it is not likely that this technical objective will be accomplished during the course of my DOD funding.

Technical objective 3: Demonstrate that the abnormal stromal microenvironment provided by CAF perturbs ESX and/or ErbB2 induction in mammary epithelial cells differently from that of heterotypic stromas; in particular, determine if CAF from ErbB2 tumors can produce and exaggerated induction in non-malignant mammary epithelial cells.

To address this question, a novel model of growing human mammary epithelium in vivo was developed (Parmar et al., 2003). This model allows the epithelial-stromal interactions to be studied which is advancement over previous models (Yang et al.,1994). Human organoids from reduction mammoplasty were combined with 250,000 mammary fibroblasts from either mouse or human origin. These were placed in a collagen gel and then grafted under the renal capsule of female nude mice for a month. The resulting grafts showed an increase in the ductal density than observed previously. These ducts expressed appropriate markers for luminal and myoepithelial cells and steroid receptors (Fig. 1 appendix). Treatment with estrogen and estrogen+progesterone resulted in an increase in ductal density and cell proliferation (Fig. 2 and 3 appendix). CAF has been combined with organoids and tested in this model. Preliminary work involved using CAF from mouse models of mammary cancer. The MMTV-Wnt-1 mouse (Tsukamoto et al., 1988) produces mammary gland tumors after 6 months. Fibroblasts from these tumors caused abnormal development of the organoids after recombination and development under the renal capsule. Also, fibroblasts from the MMTV c-neu (ErbB2) (Muller et al., 1989) mouse were utilized in this model. Organoids combined with fibroblasts from the MMTV- c-neu mouse grown under the renal capsule produced tumors, which are currently under analysis to ensure no epithelial contamination from the host.

Work to determine the effects of using CAF has been done in collaboration with Shanaz Dairkee at the California Pacific Medical Center. Dr. Shanaz is part of the breast cancer SPORE and has provided me with cells from banked tissue taken from breast cancer patients. Using organoids peripheral to the breast tumors recombined with matched CAF has resulted in abnormal epithelial development since the organoids do not grow into ducts. A panel of antibodies have been used to see expression patterns of different markers and show abnormal E-cadherin, laminin and keratin expression. Currently, the erbB2 is being tested for its expression pattern.

KEY RESEARCH ACCOMPLISHMENTS:

- Development of a novel method of growing human breast epithelium in vivo.
- Learnt renal grafting procedure
- Grown mouse embryonic mammary buds successfully under the renal capsule
- Successful tissue analysis using immunohistochemistry
- Publication of a manuscript based on work done developing in vivo model
- Manuscript in preparation on ESX data
- Invited to speak at prestigious research meetings

REPORTABLE OUTCOMES:

Invited speaker: Gordon research conference. Rhode Island 2002.

Poster and abstract. UCSF joint breast and prostate meeting. San Francisco 2002.

Poster: Gordon Research Conference. Il Ciocco. Italy 2002.

Invited speaker and poster: Era Of Hope Department of Defense breast cancer research program meeting. Orlando, Florida. 2002.

Papers in press and in preparation:

H. Parmar, P. Young, J. Emerman, R. M. Neve, S. Dairkee and G. R. Cunha. A novel method for growing human breast epithelium in vivo using mouse and human mammary fibroblasts (2002). Endocrinology 143 (12):4886-4896.

Neve R M, **Parmar H**, Mesa J and Benz C (2003).Defining the regulatory factors controlling ESX epithelial-specific expression during differentiation and cancer. Manuscript in preparation for The Journal of Biological Chemistry.

CONCLUSIONS

The implications of the in vivo mammary gland model are vast. It is now possible to manipulate both the epithelium and fibroblast components of the mammary gland to better understand mammary development with an emphasis on the human mammary gland. It also serves as a model by which to study the initial changes that occur in normal mammary epithelium when in contact with fibroblast from the tumor.

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A Novel Method for Growing Human Breast Epithelium *in Vivo* Using Mouse and Human Mammary Fibroblasts

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A novel system is described for studying the growth of normal human mammary epithelium *in vivo* as grafts in athymic nude mice. The key feature of this model is reconstitution of the epithelial-stromal interactions required for normal growth and differentiation of the human mammary epithelium, which produces ducts that are comparable to those in the normal human mammary gland. Human breast epithelial organoids were combined with mammary fibroblasts from mouse or human origin in collagen gels, which were subsequently transplanted under the renal capsule of female nude mice hosts. The resulting grafts showed an increase in the ductal density compared with that observed previously. These ducts expressed appropriate markers for luminal and myoepithelial cells and steroid receptors. Treatment of the host with diethylstilbestrol or estradiol and progesterone significantly increased the number of ducts observed and increased cell proliferation. The grafts also displayed production of β-casein and milk fat globule membrane protein when the hosts were allowed to become pregnant. This model allows for a variety of epithelial and stromal cells to be used in combination, which would aid in understanding key factors that regulate normal human mammary gland development. (*Endocrinology* 143: 4886–4896, 2002)

THE MAMMARY gland develops from ectodermal buds that invade the dermis (1). The formation of these embryonic mammary buds is induced by mammary mesenchyme. The mammary mesenchyme also determines that the ectodermal bud differentiates into ductal structures that express casein and α-lactalbumin (2). Thus, embryonic mammary mesenchyme induces the epidermis to undergo mammary differentiation, both morphologically and functionally.

After formation of the mammary epithelial bud, it elongates to form the main duct(s), which subsequently undergoes branching morphogenesis. Although ductal branching morphogenesis is initiated in many species during the prenatal period, most ductal branching morphogenesis occurs during puberty in response to estrogen stimulation (3). In the mouse the process of ductal branching begins within the developing mammary fat pad (FP) on embryonic d 17. The embryonic mammary sprout invades the underlying mammary FP precursor and branches to form a rudimentary ductal tree at birth. The FP, a deeply situated population of sc mesenchymal cells (4), contains two cell types, adipocytes and fibroblasts, and develops independently of mammary mesenchyme.

The major functional unit of the human breast is the lobular structure arising from terminal ducts. This structure consists of several small blind-ending ductules lined by a continuous layer of luminal epithelial cells surrounded by a second layer of myoepithelial cells. The myoepithelial cells are in direct contact with the basement membrane, and the duct is surrounded by fibroblasts and intralobular stroma. Stromal-epithelial interactions play key roles in mammary gland development. Tissue recombination studies have elucidated the importance of estrogen receptor α (ERα), epidermal growth factor receptor, and PTH-related protein in the stroma of the developing mouse mammary gland (2, 5, 6). Unlike that in the mouse, investigation of the *in vivo* biology of human breast epithelium (huBrE) is limited. Endocrine regulation of human breast development *in vivo* is difficult to study directly, especially when assessing the specific roles of stromal *vs.* epithelial factors. Estrogen and progesterone in cooperation with pituitary hormones are the primary systemic hormones required for the induction of proliferation and differentiation of epithelial and stromal cells leading ultimately to the formation of ductal and alveolar structures during mammary gland development. Estrogen and progesterone, acting via their specific nuclear receptors, are essential for normal mammary gland development and differentiated function. In the mouse mammary gland estrogen is important for ductal growth, and estrogen and progesterone (E2+P) action is required for alveolar development (7, 8). The action of these hormones on human mammary epithelium has proven more difficult to study than that in the mouse.

To solve the technical and ethical problems associated with the study of huBrE, investigators have employed *in vivo* models in which normal human breast tissue or isolated huBrE was transplanted into nude mice. Such grafts of huBrE alone into nude mice have produced limited outgrowth and do not take into consideration the roles of stroma and epi-

Abbreviations: DES, Diethylstilbestrol; E2+P, estrogen and progesterone; ERα, estrogen receptor α; FP, fat pad; huBrE, human breast epithelium; huBrF, human breast fibroblasts; mMgF, mouse mammary gland fibroblasts; PR, progesterone receptor; TR, tissue recombination.

thelium in hormonally regulated mammary growth and differentiation.

McManus and Welsch (9) transplanted slices of normal human breast tissue sc into athymic nude mice. Treatment of the mice with estradiol stimulated the proliferation of ductal epithelium within the grafted human breast slices (10). These studies demonstrated that when the human epithelial component and the stroma are present, various hormonal manipulations of the host animal induced proliferation. Although transplants of human breast slices have been useful for investigating hormonal parameters, such studies do not lend themselves to an examination of stromal-epithelial interactions. To assess the growth of huBrE *in vivo* under experimental conditions, normal huBrE cells were transplanted into cleared fat pads of athymic nude mice, where they formed primitive glandular structures. Unfortunately only limited outgrowth of mammary ducts has been reported. Such ducts retain normal morphology and contain myoepithelial and luminal cells (11, 12). Estrogen responsiveness was not observed despite hormone administration to the host (13). A lactational response has been demonstrated in human tissue after the administration of lactogenic hormones to the hosts or after mating the xenograft-bearing female mice (11, 14).

Disappointing initial data led to the conclusion that the mouse mammary FP was incapable of supporting expansive ductal growth of human mammary epithelium and that human and bovine mammary epithelia may require the presence of a more fibrous (collagenous) stroma than rodent mammary epithelia for proliferation and duct growth (13). In an attempt to improve ductal growth *in vivo*, human breast epithelial cells dissociated from reduction mammoplasties were embedded in extracellular matrices (type I collagen and Matrigel) before sc implantation into the nude mouse (15). Such collagen gel transplants responded to estrogen treatment (16). This system pioneered by the Nandi laboratory uses 10–30 million cells/gel. When such sc grafts were harvested, fibroblasts from the graft site had migrated into close proximity to the huBrE cells within the gel, creating an *in vivo* tissue recombination. Again, the epithelial cells were shown to have organized themselves into primitive tubules and branched structures.

Despite these encouraging results, robust ductal outgrowths have never been described in transplants of huBrE cells into nude mice. Given that the nature of the stroma is of considerable importance in determining the structure, function, and growth pattern of the resulting gland, we have modified the Nandi technique by incorporating mouse and/or human mammary fibroblasts in collagen gels containing huBrE. In so doing, a fibroblast-rich stroma is provided for the normal human breast epithelium. Instead of the relatively avascular sc graft site, our transplants are grafted under the renal capsule of nude mice. The renal site is highly vascular, thus optimizing the number of grafts successfully recovered (take rate) and growth of the graft, as previously described (17). With these modifications ductal growth is robust, with increased ductal density when huBrE is grown with either mouse or human fibroblasts. Immunohistochemical analysis of the grafts demonstrates the presence of myoepithelial cells and luminal epithelial cells that express appropriate steroid receptors. A response to hormonal stimuli was observed after treatment of the host with DES or E2+P pellets. A lactogenic response, induction of β-casein and milk fat globule protein, has also been shown in the grafts grown in pregnant hosts. This improved method of growing huBrE *in vivo* allows study of mammary development and elucidation of the role of stromal-epithelial interactions during growth and differentiation of human mammary epithelium *in vivo*.

Materials and Methods

Tissue sources

Human breast tissue, consisting of reduction mammoplasty specimens from five patients, was obtained from clinics at University of British Columbia (Vancouver, British Columbia, Canada). Patients were between 20 and 32 yr of age. Normal human mammary fibroblasts were obtained from both University of British Columbia and the California Pacific Medical Center (San Francisco, CA). This work was undertaken with institutional review board approval from all institutions involved in the study.

Hoechst 33258 dye staining

Formalin-fixed paraffin sections were stained with 4 μg/ml Hoechst 33258 (Calbiochem, La Jolla, CA) for 1 min at room temperature. This was followed by 1–5 min of washing under running tap water.

Production of human breast epithelial organoids and fibroblasts

Normal tissue from reduction mammoplasties was finely minced with scalpels and transferred to a dissociation flask (Bellco, Vineland, NJ) containing DMEM/Ham's F-12 supplemented with 10 mM HEPES, 2% BSA (fraction V), 300 U/ml collagenase type 1, and 100 U/ml hyaluronidase. After 18 h at 37 C on a shaker, the resulting suspension was centrifuged at 40 × *g* for 30 sec to pellet epithelial cell clumps and organoids. This pellet was washed twice with fresh DMEM/Ham's F-12 to remove residual enzyme, stromal cells, and tissue debris.

Alternatively, the suspension was filtered through a 51-μm pore size nylon filter to separate organoids that were retained on the filter from a fibroblast-enriched filtrate as described previously (18). Actively proliferating fibroblast cultures were maintained in DMEM/Ham's F-12 and 10% fetal bovine serum.

Production and growth of mouse mammary fibroblasts

Number 4 mammary FPs were removed from 3-wk-old female wild-type C57/B6 mice. The medial portion containing the mammary duct was excised and discarded. The remaining lateral portion of the FP was chopped finely with a razor blade in a minimal amount of medium (DMEM/Ham's F-12 + 10% fetal bovine serum) in a petri dish. Ten milliliters of medium were added to the dish, and the chopped tissue was transferred to a small 25-ml conical flask. After the addition of 10 U/ml collagenase (Sigma, St. Louis, MO), the mince was incubated for 20 min at 37 C. The tissue was then centrifuged for 10 min at 200 × *g*, the supernatant (containing fat) was removed, and the pellet was resuspended in 5 ml fresh medium. The mixture was centrifuged at 200 × *g* for 2 min, the supernatant was removed, and the pellet was resuspended in 5 ml medium. The final spin was for 30 sec, after which the pellet was resuspended in 1 ml medium and plated into a 75-ml flask. The resultant cultures were predominantly fibroblastic and negative for keratin expression.

Epithelium and fibroblast recombinations

About 6–8 organoids of huBrE (~50,000–100,000 cells) were mixed with 250,000 fibroblasts in 20 μl neutralized collagen. After polymer-

ization and overnight culture at 37 C, the collagen gels containing normal huBrE and fibroblasts were grafted under the renal capsule of female nude mouse hosts. Grafts were harvested after 1 month.

Animals

Forty- to 50-d-old female CD-1 nude mice were used as hosts for the grafts (Charles River Laboratory, Wilmington, MA). All food, water, cages, and bedding were sterilized before use. Aseptic techniques were used at all times for handling the mice. Renal grafting was carried out in laminar flow hoods. Before renal grafting, recipient hosts were anesthetized with Avertin at 0.02 ml/g body weight. A total of 40 mice were used in this study, and grafts were left in the animals for 1 month. The studies were carried out with approval from the University of California-San Francisco committee on animal care.

Renal grafting

The process of renal capsule grafting is described and illustrated in detail at the following website: http://mammary.nih.gov/tools/mousework/Cunha001/index/html.

Grafts were harvested after 1 month and could be identified as a white mass ranging from 0.25–0.5 cm in length on the kidney. After removal, the grafts were fixed overnight in 10% phosphate-buffered formalin (Fisher Scientific, Fairlawn, NJ), embedded in paraffin, sectioned, and stained by hematoxylin and eosin using standard procedures.

Hormone treatment

All pellets were made using a Parr pellet press. Mice were treated with a diethylstilbestrol (DES) pellet (2 mg DES and 18 mg cholesterol) for 30 d continuously from the time of grafting. Mice were also treated with 10 mg E2+P pellets (1:1000, E2:P). The pellets were placed sc at the time of grafting.

Immunohistochemistry

Histological examination was performed after fixation in 10% formalin, paraffin embedding, and sectioning at 6 μm. The immunohistochemical techniques used have been described previously (19). The antibodies used were mouse monoclonal keratins 8 and 14 (provided by Dr. Birgitte Lane, University of Dundee, Dundee, Scotland), Ki67 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ERα (DAKO Corp., Carpenteria, CA), PR (DAKO Corp.), human milk fat globule (Accurate Chemical and Scientific Corp., Westbury, NY), β-casein (Harlan Sera-Lab, Loughborough, UK). The avidin-biotin-peroxidase procedure (20) was followed using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA).

Mammary gland extraction and immunoblotting

Mammary glands from virgin and pregnant mice and huBrE and mouse mammary gland fibroblasts (mMgF) grafts from virgin and pregnant hosts were lysed in Nonidet P-40 lysis buffer [50 mM HEPES (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 25 mM β-glycerol phosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 μg/ml leupeptin and aprotinin, and 1 mM phenylmethylsulfonylfluoride], and tissue was pelleted at 10,000 $\times$ *g* for 30 min. Mammary gland and graft extract protein concentrations were determined by the method of Bradford, and protein was separated on 7.5–15% SDS-PAGE gels, blotted onto polyvinylidene difluoride, and detected by enhanced chemiluminescence as described previously (21).

Results

Human breast reduction specimens were collagenase-digested to yield ductal organoids (small segments of ducts). Six to eight organoids (50,000–100,000 cells) were mixed with 250,000 mammary fibroblasts in 20 μl collagen and then grafted under the renal capsule of female nude mouse hosts. Two of the gels containing organoids and fibroblasts were placed under the renal capsule. Organoids were used from five separate female patients between 20–32 yr of age. The take rate of the grafts was calculated as the number of grafts successfully recovered from the number of tissue recombinations (TRs) inserted under the renal capsule. Successful grafts were shown by histology to contain epithelium and ductal structures. The overall take rate of grafted tissue recombinations was 80%. The take rate and ductal density were higher when mouse mammary fibroblasts were used than when human mammary fibroblasts were used.

Freshly prepared human breast organoids

Freshly prepared human breast ductal organoids before grafting retained little ductal morphology, and immunostaining with antibodies to myoepithelial and luminal cell markers demonstrated the presence of both of these cell types in the collagenase-isolated ductal organoids (data not shown). Histological analysis of the organoids showed a lack of contaminating human stroma. Thus, the organoids were essentially free of connective tissue elements.

HuBrE grafted alone in the collagen gel

Organoids transplanted in collagen gel without the addition of fibroblasts formed small ducts and solid epithelial cords after 1 month of growth in intact female nude hosts (Fig. 1). Typically, only three ductal profiles were observed per microscopic field using a $\times$20 objective lens. These epithelial structures were surrounded by fibroblasts. Hoechst dye was used to determine the nature of the surrounding fibroblasts (22). Mouse fibroblasts were shown to contain intranuclear bodies and were distinguishable from human fibroblasts. Based on this Hoechst dye staining, the fibroblastic stroma was overwhelmingly of mouse origin (data not shown). Only a few human fibroblasts were detected, indicating that fibroblastic contamination of the organoids was minimal. The fibroblasts were presumably mouse renal fibroblasts from the graft site that had invaded the collagen gel. The epithelial structures and cords contained luminal cells expressing keratin 8 and myoepithelial cells expressing keratin 14 and smooth muscle actin. The ductal structures also expressed low levels of ERα and very low almost undetectable levels of the progesterone receptor (PR). DES administration to the host resulted in an increase in the number of ductal structures observed to an average of five per microscopic field using a $\times$20 objective lens. There was also loss of ERα expression and induction of PR (Fig. 1). Anti-Ki67 antibody, which recognizes a human nuclear cell proliferation-associated antigen, was used as the marker for proliferation. The Ki67 labeling index increased by 100% after treatment with DES relative to that observed in the intact or ovariectomized host. This means that cell proliferation increased 2-fold in response to DES even in transplants of epithelium alone (Fig. 2B).

To provide an appropriate stromal environment for the human breast epithelium, mMgF or normal human breast fibroblasts (huBrF) were incorporated into the collagen gel before grafting. This technical modification lead to increased ductal growth. There was an average of eight ductal profiles

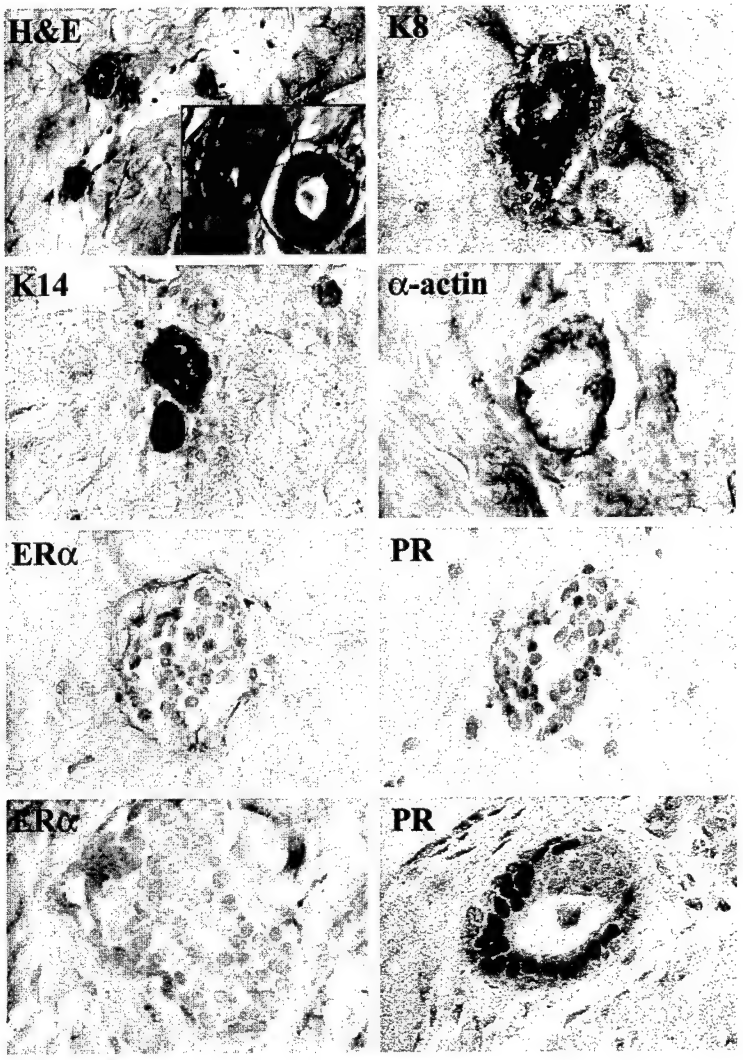

FIG. 1. Histology and DES response of organoids grown under the renal capsule for 1 month. Hematoxylin-eosin staining of organoids grown under the renal capsule for 1 month in an untreated host, shown at ×100 (*inset* at ×400) magnification. The small epithelial cell structures are positive for keratins 8 and 14 (K8 and K14) and α-actin. Low levels of ER and PR are observed in the untreated organoids. Organoids harvested from mice treated with a sc DES pellet display a hormonal response, as ER expression is down-regulated, and PR is induced. Immunohistochemistry sections are shown at ×400 magnification.

per microscopic field (×20 objective lens) in huBrE and mMgF grafts from an intact untreated host. Addition of mMgF to the organoids led to a 3-fold increase ($P = 0.0105$) in the number of ducts (Fig. 2A). The ducts observed in both huBrE+huBrF and huBrE+mMgF tissue TRs contained a continuous layer of luminal cells surrounded by a discontinuous layer of myoepithelial cells and resembled normal human breast tissue (Fig. 3). Ducts of huBrE+huBrF and huBrE+mMgF recombinations demonstrated the expression of keratin 8 in luminal epithelial cells. Myoepithelial cells expressed keratin 14 and α-smooth muscle actin, as is the case for normal human breast epithelium (Fig. 3). In the normal human adult breast, ERs are expressed in an average of 7% of luminal epithelial cells (23–25) and are undetectable in the stroma. Eighty to 100% of the luminal epithelial cells in huBrE+mMgF and huBrE+huBrF recombinations expressed ERα in untreated intact or ovariectomized female hosts (Fig. 4, A and B). PR was detected at low levels in huBrE+huBrF and huBrE+mMgF TRs grown in untreated intact hosts (Fig. 4, A and B). Thus, histodifferentiation of huBrE was comparable to that in normal human breast tissue when grown in association with normal human breast or mouse mammary gland fibroblasts. Fibroblasts from both sources allowed for the normal differentiation and robust ductal development of the huBrE *in vivo* after 1 month under the renal capsule.

DES-induced proliferation of huBrE grown in association with mouse and human mammary fibroblasts

Tissue recombinations prepared with huBrE+huBrF or huBrE+mMgF were treated continuously for 30 d by sc DES pellet. Hematoxylin-eosin staining showed an increase in the number of ducts observed in DES-treated grafts of both huBrE+mMgF (Fig. 4A) and huBrE+huBrF (Fig. 4B). In the untreated intact or ovariectomized host, an average of 8 ducts were observed/microscopic field at ×20 objective, whereas an average of 11 ducts/microscopic field was observed in huBrE+mMgF treated with DES. This significantly increased the ductal number (Fig. 2A). Ki67 epithelial labeling was higher in the ducts in huBrE+mMgF after the addition of fibroblasts than after grafting the organoids alone in intact untreated hosts (Fig. 2B), although this increase was not statistically significant. This suggests that incorporation of mouse or human mammary fibroblasts into the collagen gels enhances the pro-

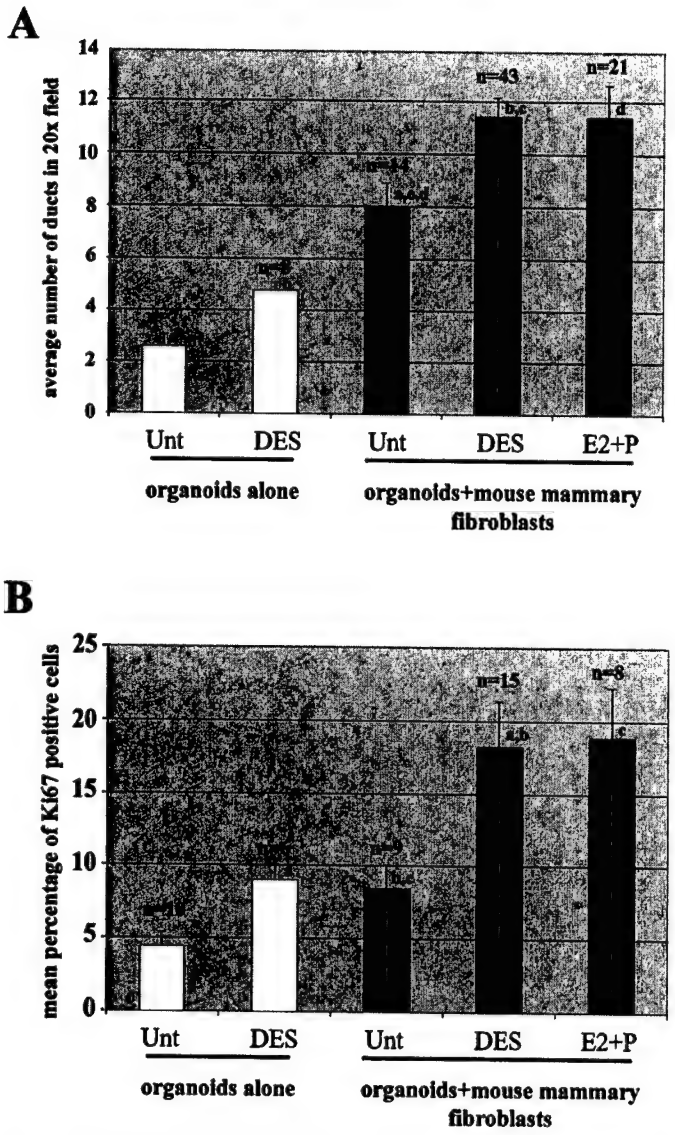

FIG. 2. Comparison of ductal numbers and epithelial Ki67 labeling indexes from grafts of untreated and hormone-treated organoids alone and with huBrE+mMgF. A, Comparison of ductal densities of untreated and hormone-treated grafts of organoids alone and huBrE+mMgF tissue recombinations using a ×20 objective: a, untreated organoids when combined with fibroblasts showed a significant increase in ductal numbers ($P = 0.0105$); b, DES treatment of organoids and mouse fibroblasts significantly increased ductal numbers relative to those in DES-treated organoids alone ($P = 0.0002$); c, DES treatment of organoids and mouse fibroblast recombinations significantly increased ductal numbers relative to untreated values ($P = 0.0152$); d, E2+P treatment of organoids and mouse fibroblast recombinations significantly increased ductal density relative to that in the untreated group ($P = 0.0152$). B, Ki67 labeling index observed using a ×20 objective: a, DES-treated organoids and mouse fibroblasts significantly increased Ki67 relative to DES-treated organoids alone ($P = 0.0257$); b, DES-treated organoids and mouse fibroblasts significantly increased the Ki67 index relative to that in the untreated group ($P = 0.0075$); c, E2+P treatment of organoids and mouse fibroblasts significantly increased Ki67 labeling relative to that in the untreated group ($P = 0.0132$)

liferation of huBrE cells. This effect on epithelial proliferation coincided with an increase in the number of ducts per microscopic field in grafts combined with mammary fibroblasts. The Ki67 labeling index was increased by 2-fold in huBrE+mMgF tissue recombinations from DES-treated hosts relative to that in grafts of organoids only grown in DES-treated hosts. The number of ducts per microscopic field in huBrE+mMgF increased in parallel with the Ki67 labeling index from 8 to an average of 11 after DES treatment. The huBrE+huBrF recombinations showed a similar increase in Ki67 labeling and ductal density after DES treatment (Fig. 4B). Treatment of hosts continuously with DES by sc pellet for 30 d induced a profound down-regulation of epithelial ERα in huBrE grown in association with mouse or normal human mammary fibroblasts. PR was induced by DES treatment in luminal epithelial cells of huBrE+mMgF and huBrE+huBrF tissue recombinations (Fig. 4, A and B). Thus, both types of fibroblasts provide a favorable stromal environment for the growth of human mammary epithelium and allow for estrogenic response.

E2+P induced proliferation of huBrE grown in association with mouse mammary fibroblasts

Tissue recombinations were prepared with huBrE+mMgF and treated continuously for 30 d with 10 mg E2+P pellets in intact or ovariectomized hosts. The E2+P was administered by sc pellet as described by Popnikolov and colleagues (15), which produces systemic levels of about 250 pg/ml E2 and 40 ng/ml P (both ~10-fold above normal). This hormonal treatment has been shown to stimulate the proliferation of grafted huBrE (16). For the most part the effects of E2+P were identical in most aspects to those of treatment with DES alone. Treatment of huBrE+mMgF grafts with E2+P down-regulated epithelial ERα (Fig. 4A) and stimulated epithelial proliferation. The epithelial Ki67 labeling index in E2+P-treated grafts was increased more than 2-fold (from 8 to 18 cells labeled) compared with that for untreated tissue recombinations (Fig. 2B). In parallel, E2+P increased ductal density to an average of 11 ducts/microscopic field, similar to that observed with DES (Fig. 2A). There was no significant increase in Ki67 or ductal density by combining progesterone with estrogen. E2+P did not induce epithelial PR expression as was observed with DES (Fig. 4A).

Effect of host pregnancy on huBrE+mMgF recombinations

For the human breast it has been difficult to study the roles of various hormones and growth factors on the proliferation of epithelial cells during pregnancy. Many endocrinological changes occur during pregnancy, including variations in estrogen, progesterone, glucocorticoid, PRL, and placental lactogen. Due to the complexity of the endocrine profile in pregnancy it has been difficult to mimic pregnancy conditions in organ culture or primary cell culture. *In vivo*, a lactogenic response has been shown previously in organoids injected into the mouse mammary gland of nude mice after host matings (14). To elicit a lactogenic response in the grafts of huBrE grown in association with mouse or human mammary fibroblasts, the host mice were mated 3 wk after grafting. The grafts were removed before delivery of the pups at about 18 d gestation. Histological analysis of the ducts showed a clear lactogenic response. Ducts were distended

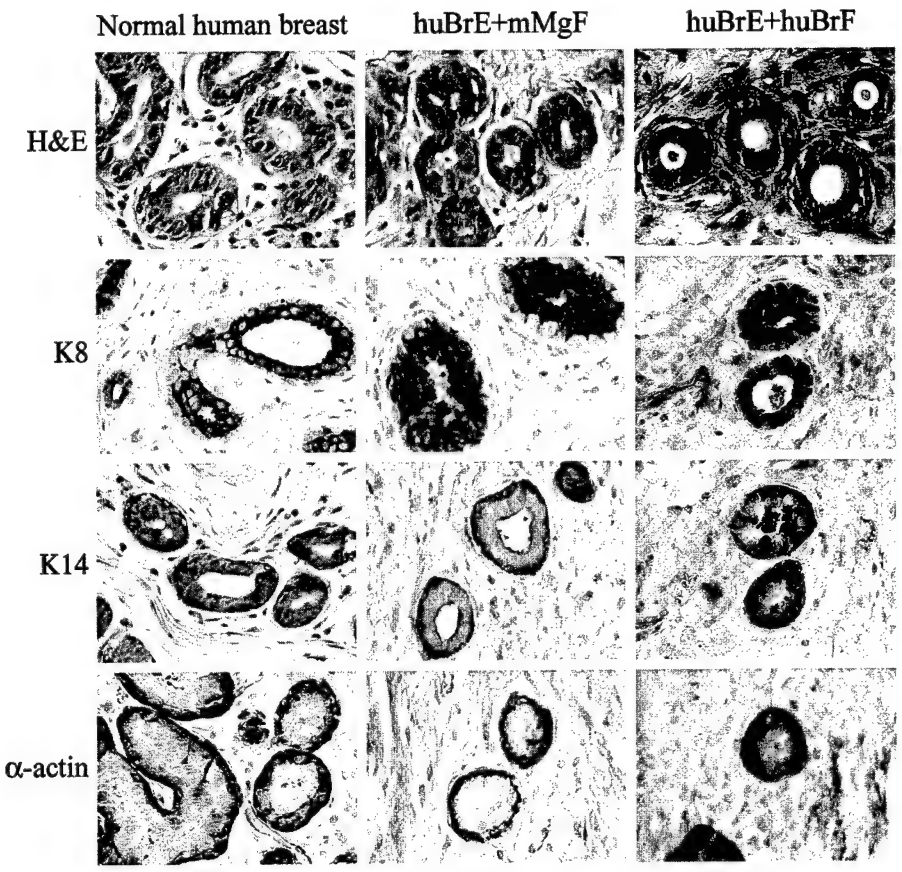

FIG. 3. Histology and differentiation marker expression in human mammary ducts and ducts produced from huBrE+mMgF and huBrE+huBrF tissue recombinations grown for 1 month under the renal capsule. Hematoxylin-eosin staining of ducts from normal human breast and ducts from huBrE+mMgF and huBrE+huBrF all show the presence of ducts with lumen. Immunohistochemistry with differentiation markers for luminal epithelial cells [keratin 8 (K8)], myoepithelial cells [keratin 14 (K14)], and smooth muscle actin (α-actin) display similar expression patterns in normal breast and ducts from the tissue recombinations. All images are shown at ×400 magnification.

with secretions, and the apical cytoplasm of luminal cells was vacuolated (Fig. 5A).

Confirmation of the lactation response in huBrE+mMgF recombinations was obtained using mouse monoclonal antibodies against β-casein and the milk fat globule membrane protein antigen. The results showed an increase in the expression of β-casein and the fat globule compared with grafts harvested from nonpregnant hosts (Fig. 5B). Western analysis of virgin and pregnant mouse mammary glands and huBrE+mMgF grafts from virgin and pregnant hosts was also carried out (Fig. 5C). Using an antibody against human β-casein gave a specific and a nonspecific band in the pregnant mouse mammary gland sample (Fig. 5C, lane 2). A significant increase in β-casein was observed in huBrE+mMgF recombinations harvested from a pregnant host, where a single specific band of 28 kDa was observed (Fig. 5C, lane 4). This demonstrates the ability of the graft to undergo changes in morphology and function associated with pregnancy.

Discussion

The goal of our work was to develop, characterize, and validate a new *in vivo* model of studying normal human breast epithelium. Our model is unique in that it takes into account the stromal-epithelial interactions that occur in the mammary gland. The addition of human or mouse fibroblasts to huBrE facilitates normal human mammary development, producing ductal structures analogous to those of the normal human mammary gland that are hormone responsive and able to undergo functional differentiation. This model can now be used to study the effect of genetically modified human or mouse stroma on human mammary epithelium.

Our studies of the growth of huBrE in nude mice differ from all previous reports in several respects. Our experiments are the first to graft mixtures of huBrE plus fibroblasts (not adipose tissue) as the stromal element, and thus differ from previous reports of injecting huBrE cells into mouse cleared FPs. Based upon previous transplantation experiments, the stromal requirements of mouse and human mammary epithelium are species specific. Mouse mammary gland epithelium undergoes extensive growth and ductal branching in an adipose stroma provided by the mammary FP. In contrast, human mammary epithelium transplanted into the mouse mammary FP does not grow, but, rather, forms rounded hollow structures encapsulated by connective tissue (13, 15, 26). Although exogenous hormones stimulated DNA synthesis in these structures, expansive ductal outgrowth of the huBrE did not occur (13). Almost identical morphological and proliferative responses were recorded when primary bovine mammary epithelial cells were transplanted into the mouse mammary FP, strongly suggesting that the stromal component provided by the mouse mammary FP is not optimal for ductal growth and branching of both human and bovine mammary epithelial tissues (27). Sheffield suggested that "human mammary epithelia may require the presence of a more fibrous (collagenous) tissue than rodent mammary epithelia for normal proliferation" (13). Thus,

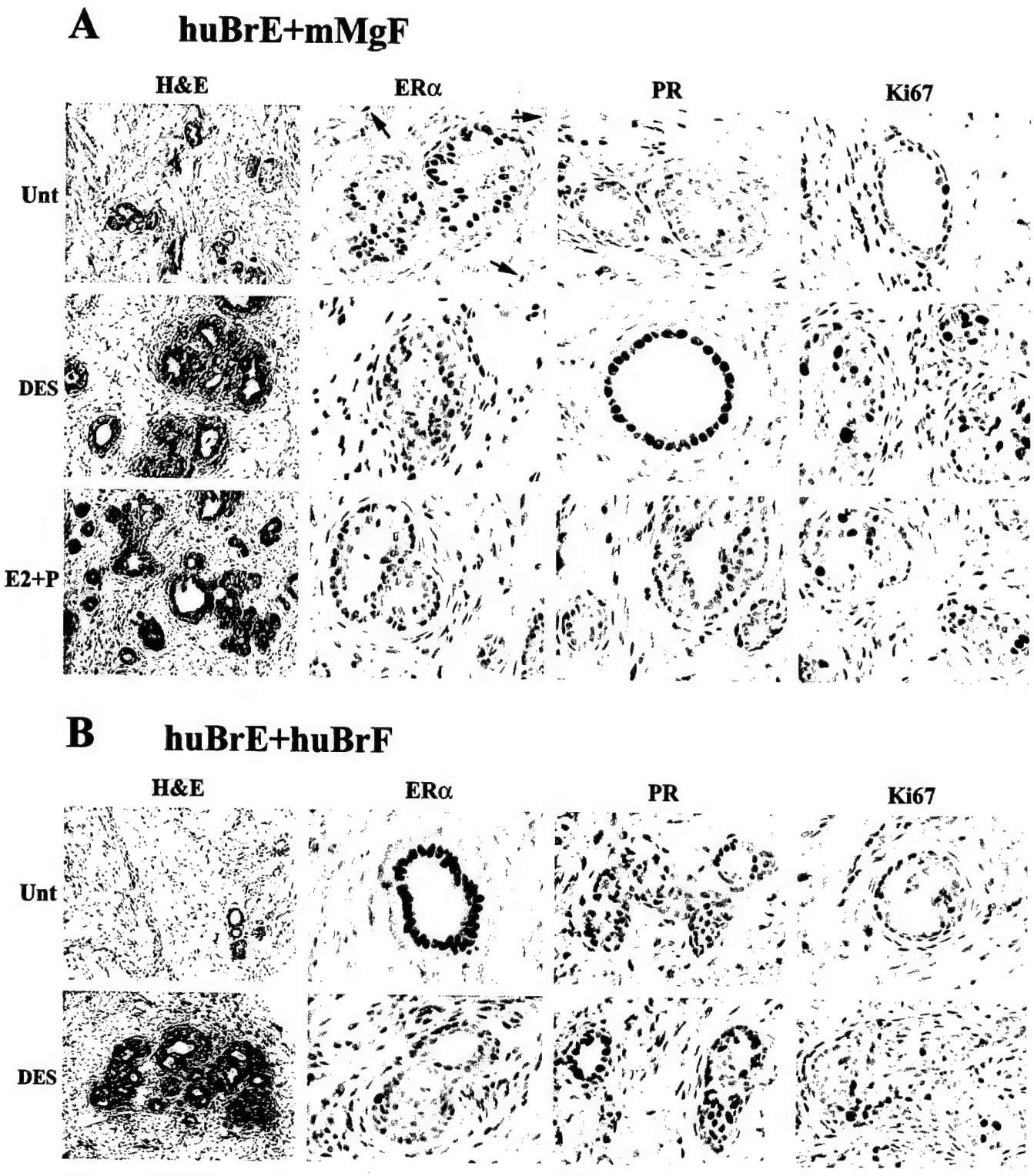

FIG. 4. Hormonal and proliferation responses in huBrE+mMgF and huBrE+huBrF tissue recombinations. Ducts from huBrE+mMgF (A) increase in number after treatment with DES and E2+P, as shown by hematoxylin-eosin staining. Untreated grafts are ERα positive and have very low levels of PR and few Ki67-positive cells. Treatment with DES for 1 month resulted in an increase in ductal density, down-regulation of ER, and induction of PR. There was also an increase in the Ki67 labeling index. Treatment with E2+P for 1 month resulted in an increase in ductal density, down-regulation of ER, and no induction of PR, but a similar increase in Ki67 labeling as that observed with DES treatment. *Arrows* indicate ERα-positive fibroblasts in the stroma. B, huBrE+huBrF tissue recombinations also show an increase in ductal numbers after treatment with DES, as shown by hematoxylin-eosin staining. Ducts from untreated grafts were ERα positive and had low levels of PR and Ki67-labeled cells. Treatment with DES pellet for 1 month resulted in an increase in the ductal density and down-regulation of ER. PR was induced, and there was an increase in the Ki67 labeling index. Images are shown at ×100 magnification with hematoxylin-eosin staining. ERα, PR, and Ki67 are shown at ×400 magnification.

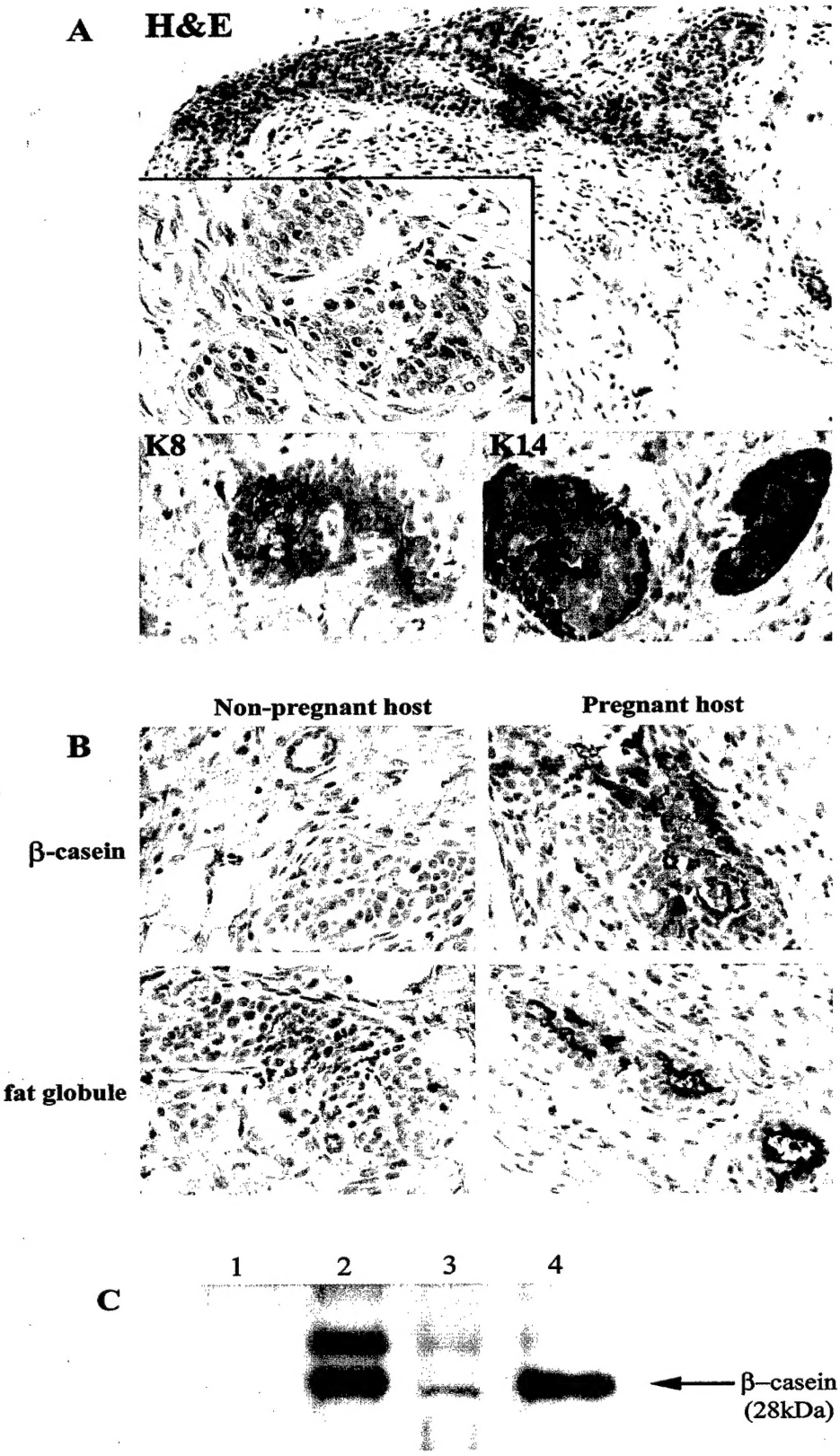

FIG. 5. huBrE+mMgF recombinations undergo morphological change and functional differentiation in the pregnant host. A, Hematoxylin-eosin staining at low power (×100) shows stimulation of ducts under the influence of the host pregnancy state. At high power, ducts are observed to be vacuolated and distended with secretions and are keratin 8 (K8) and keratin 14 (K14) positive (×400 magnification). B, In untreated hosts, immunostaining for antihuman β-casein is undetectable, and milk fat globule expression is weak. In grafts harvested from an 18-d-pregnant host, β-casein and milk fat globule are strongly expressed (×400 magnification). C, Western blot analysis of β-casein protein using a human-specific antibody shows an increase in huBrE+mMgF recombinations harvested from 18-d-pregnant hosts compared with grafts from nonpregnant mice. Virgin mouse mammary gland (lane 1) has no β-casein expression. Pregnant mouse mammary gland (lane 2) shows two bands at 28 and 32 kDa. huBrE+mMgF recombination from virgin host (lane 3) shows very weak expression, and huBrE+mMgF from pregnant host shows a single specific band at 28 kDa (lane 4).

combining the mammary epithelium with mammary stromal fibroblasts may more appropriately reconstitute the normal stromal environment of the human mammary gland.

Use of the renal capsule as a graft site is another technical modification relative to previous work. The vascularity of the renal site far exceeds that of the sc site and dramatically increases cellular survival in the first 24 h

postgrafting. This maximizes the effective inoculum size and take rate. The effective inoculum size is the number of cells surviving the anoxic conditions in the first 24–48 h before vascularity of the graft is established. During this critical period, a certain percentage of cells die, thus decreasing the effective inoculum size. The percentage of surviving cells is then available to generate mammary ductal tissue. The number of cells lost immediately postgrafting can be exceedingly high, especially in the relatively avascular sc graft site. We see this advantage of the renal graft site in our current mammary studies insofar as the number of ductal profiles observed when 50,000–100,000 huBrE cells (6–8 organoids) are transplanted with fibroblasts appears to be more than or equal to that observed when 10–30 million huBrE cells are transplanted sc in collagen gels (12).

Most important we have verified that human or mouse mammary fibroblasts can support normal histodifferentiation and functional differentiation of human breast epithelium. The normal human mammary gland shows a double-layered epithelium consisting of a single layer of columnar luminal cells and a basal cell layer of myoepithelial cells. Xenografted organoids and mammary fibroblasts also showed this organization, as luminal cells were positive for keratin 8, and the basal cells were positive for keratin 14 and α-actin. From the huBrE+mMgF and huBrE+huBrF tissue recombinations analyzed, TRs containing mouse mammary fibroblasts consistently gave better results than TRs containing human fibroblasts, as the grafts were larger, the take rate was higher, and more ductal structures were observed per microscopic field. The mouse mammary fibroblasts were derived from female donors 3 wk of age; therefore, the fibroblasts were young and from growing mammary gland. The human fibroblasts were isolated from reduction mammoplasty patients and thus were derived from mature glands. This factor may have influenced the growth and developmental potential of the human mammary ducts. Ducts in both huBrE+huBrF and huBrE+mMgF TRs were DES responsive, and after the host was mated, milk proteins were detected in the ductal lumen. The hormone response is a key feature of this model, as ducts behave similarly to the normal human mammary gland.

The organoids when transplanted alone under the renal capsule, *i.e.* without addition of fibroblasts, were DES responsive, as shown by a 2-fold increase in the Ki67 labeling index and an increase in the ductal density per microscopic field. This suggests that human mammary epithelium alone is able to respond directly to DES. Combining the organoids with fibroblasts increased the number of ducts per microscopic field and increased the percentage of proliferating cells (in untreated hosts), providing a more optimal condition for growth of the epithelium. After treatment with the DES pellet for 1 month, epithelial ERα was down-regulated, and epithelial PR was induced, confirming previous reports of huBrE grafted alone in collagen gel (28). These effects on ERα and PR were observed when the organoids were implanted alone or in combination with mouse or human mammary fibroblasts. In the huBrE+mMgF tissue recombinations grown in intact untreated hosts, ERα was detected in both the epithelial and stromal compartments. In huBrE+huBrF TRs grown in an intact untreated host, ERα was detected only in the luminal epithelium and not in the stroma. Eighty percent of luminal huBrE cells were ERα positive, which is vastly higher than the 7% found in normal adult human breast tissue (29, 30). This may be due in part to the lower systemic levels of estrogen found in the nude mouse *vs.* the adult human. As estrogen down-regulates ERα, the lower systemic estrogen levels provided by the nude mouse host may allow more of the epithelial cells to express ERα. Alternatively, the increased number of ERα-positive cells may be due to the temporary reversion of the epithelium to a more primitive developmental state when ERα expression is known to be much higher (30). Administration of DES via sc pellet at the time of grafting resulted in a down-regulation of the ERα and an induction of the PR in huBrE+mMgF and huBrE+huBrF TRs. The reduction in ERα levels and the simultaneous induction of PR levels after treatment with estrogen have also been observed in human breast tissue implanted into female intact nude mice treated with estrogen (31). This is probably a direct effect of DES on the mammary gland epithelium not requiring a specific stromal environment. ER also has important interrelationships with the PR system in the modulation of biological responses. PR expression is regulated by estrogen via estrogen response elements on the progesterone receptor (32). By binding of estrogen to progesterone and estrogen response elements, the PR is induced. In most tissues coexpressing PR, estrogen controls the regulation of PR, thereby also controlling sensitivity to progestins. Thus, PR expression is up-regulated by estrogen and down-regulated by progesterone in most target tissues (33), including huBrE grown *in vivo* in combination with mMgF or huBrF. Administering both estrogen and progesterone to huBrE+mMgF at the time of grafting also showed a proliferative response. However, although DES alone induced PR, epithelial PR was undetectable in huBrE+mMgF tissue recombinants treated with E2+P. The regulation of PR gene expression is complex. In various cell types PR is induced via different mechanisms. For many cell types PR is directly induced by estrogens in the responding cells and decreased by progestins. However, in mouse uterine epithelium PR is constitutively expressed in the absence of signaling through ERα. Treatment with E2 down-regulates uterine epithelial PR, an effect mediated through ERα in the stroma (34). PR is also up-regulated in the mouse uterus as a result of decidualization through mechanisms not understood (34), but not involving ERα. The mechanism of down-regulation of epithelial PR in huBrE+mMgF TR treated with E2+P clearly involves the action of P. This may involve the direct action of P on the mammary epithelial cells. Alternatively, paracrine activity may be involved. Ongoing and planned experiments using mouse mammary fibroblasts from ERKO and PRKO mice may resolve this issue. In the context of the assays performed in the present study, the induction of PR was the only end point that distinguished the effect of DES alone from that of E2+P. The Ki67 index and the ductal profiles of huBrE+mMgF and huBrE+HuBrF TRs treated with DES alone or E2+P were virtually identical, with no additive effect of the E2+P.

The induction of human casein in the huBrE+mMgF grafts after 18 d of mouse pregnancy demonstrates the ability of the human breast epithelium to undergo the complex morphological and differentiation changes that occur in the normal mammary gland. A variety of endocrinological changes occur during pregnancy, including

variations in estrogen, progesterone, glucocorticoid, PRL, and placental lactogen. Histological analysis of grafts grown in pregnant hosts revealed distended vacuolated epithelial cells and secretions in ductal lumen. Mammary epithelial cells grafted in collagen gels do not exhibit this response under similar conditions (35), suggesting that the specific reconstituted stroma employed in this study facilitate functional differentiation. Functional differentiation culminated in increased expression of β-casein protein, as shown by Western analysis. The lactation observed in the huBrE+mMgF recombinations was a response exclusively to murine hormones exerting their influence over the 3-wk span of a murine pregnancy. Although the hormonal conditions provided by the mouse host were probably not optimal, it is evident that human mammary epithelium is responsive to mouse PRL. These results are consistent with studies using organoids injected into the nude mouse (14).

The results show that huBrE develops, grows, responds to hormones, and differentiates in association with normal mouse and human mammary gland fibroblasts when grafted under the renal capsule in a collagen gel. The similarity in results of using both human and mouse stroma demonstrate that processes in the human breast are sufficiently identical to those in the mouse so that normal mMgF can substitute for human mammary stroma. Because wild-type mouse mammary fibroblasts facilitate or promote the growth and development of human breast epithelium, future studies can use mouse mammary fibroblasts derived from a variety of knockout mice to assess the roles of various paracrine pathways in growth, differentiation, and hormone response of huBrE. Also, experimental knockouts of genes in human fibroblasts can be used. Using this methodology, it will be possible to address the effects of specific stromal factors on human mammary gland development *in vivo*. This novel approach provides an excellent model in which to study the development and epithelial-stromal interactions that take place in the development, growth, and differentiation of human mammary epithelium. The use of tumor fibroblasts or tumorigenic epithelium in this model will also allow for the study of breast cancer.

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